

P-Selectin and Vascular Cell Adhesion Molecule 1 Mediate Rolling and Arrest, Respectively, of CD4⁺ T Lymphocytes on Tumor Necrosis Factor α -activated Vascular Endothelium under Flow

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Summary

This report examines the adhesive interactions of human CD4⁺ T lymphocytes with tumor necrosis factor α -activated human endothelial cell monolayers in an in vitro model that mimics microcirculatory flow conditions. Resting CD4⁺ T cell interactions with activated endothelium consisted of initial attachment followed by rolling, stable arrest, and then spreading and trans-endothelial migration. P-selectin, but not E-, or L-selectin, mediated most of this initial contact and rolling, whereas β_1 -integrins ($\alpha_4\beta_1$), interacting with endothelial-expressed vascular cell adhesion molecule 1, participated in rolling and mediated stable arrest. In contrast, β_2 -integrins were primarily involved in spreading and transmigration. These findings highlight an important role for P-selectin and suggest discrete functions for β_1 - and β_2 -integrins during lymphocyte recruitment to sites of immune-mediated inflammation.

Peripheral blood CD4⁺ lymphocyte adhesive interactions with vascular endothelium are essential for normal immune function and for recruitment to sites of inflammation. Previous in vitro studies (1–4) have demonstrated that T cell adhesion to cultured endothelial cells under static conditions is mediated by multiple adhesion pathways and is dependent on the state of activation of both cell types. For resting CD4⁺ cell adhesion to activated endothelial cells, at least three separate receptor-ligand pairs are involved: intercellular adhesion molecule 1 (ICAM-1)–LFA-1; vascular cell adhesion molecule 1 (VCAM-1)–very late antigen 4 (VLA-4) ($\alpha_4\beta_1$); E-selectin–sialyl Lewis^x, and/or related carbohydrates on leukocytes. Recent studies in vivo or in vitro under flow conditions have revealed that multiple receptor-ligand pairs can act sequentially and in an overlapping manner to effect leukocyte initial attachment, rolling, stable arrest, and ultimately, emigration (4). Current models propose that members of the selectin gene family (E-, P-, and L-selectin) mediate the initial adhesive interactions, termed leukocyte “rolling” (4–11) or “tethering” (12) and that subsequent firm adhesion and diapedesis requires activation-dependent engagement of integrins with their endothelial ligands, and CD31 (platelet/endothelial cell adhesion molecule 1 [PECAM-1]) (13), respectively. These models are based on observations with neutrophils and monocytes, and the extent to which lymphocytes

follow this paradigm remains untested. Using an in vitro flow chamber to simulate flow conditions likely to exist in post-capillary venules (14) and function-blocking mAbs, we studied the cellular processes and molecular events involved in CD4⁺ T cell adhesion to TNF- α -activated human umbilical vein endothelial cell (HEC) monolayers.

Materials and Methods

Endothelial Cell Cultures, CD4⁺ T Lymphocytes, and Murine-transfected Cell Lines. HECs were isolated and cultured as previously described (15). Confluent HEC monolayers were treated with or without TNF- α (recombinant human, 50 ng/ml; Genentech, Inc., San Francisco, CA) in M199-10% FCS for 6 h at 37°C before immunofluorescence staining or flow chamber studies. Chinese hamster ovary (CHO) cells expressing a stably transfected human P-selectin cDNA (CHO P-selectin) were obtained from Dale Cumming (Genetics Institute, Cambridge, MA). L-cells expressing a stably transfected human E-selectin cDNA or a seven-domain form of human VCAM-1 cDNA were obtained from Thomas F. Tedder (Duke University, Durham, NC). Transfected L cells were cultured in DMEM containing 10% FCS, antibiotics, L-glutamine, and minimal essential amino acids (5). CD4⁺ T cells were purified from single donor human platelet pheresis residues by sequential density gradient centrifugation and elutriation, followed by culture overnight and then positive selection on Dynabeads (model M-450 CD4) and DETACHaBEAD (Dynal, Great Neck, NY).

mAb Reagents. Function blocking anti-L-selectin antibodies LAM1-3 and LAM1-4, and nonblocking anti-L-selectin antibody LAM1-14 (all IgG1) were obtained from Thomas Tedder (16). Blocking anti-L-selectin antibody Dreg-56 was obtained from Eu-

¹ Abbreviations used in this paper: HEC, human umbilical vein endothelial cell; VCAM-1, vascular cell adhesion molecule 1.

gene Butcher (Stanford University, Stanford, CA). These anti-L-selectin antibodies were used as purified IgG at 10 $\mu\text{g/ml}$. Blocking and nonblocking anti-P-selectin antibodies, HDPG 2/3 and HPDG2/1 (both IgG1), respectively, were obtained from Dale Cummings (17) and were used as purified IgG at 10 $\mu\text{g/ml}$. Blocking and nonblocking anti-VCAM-1 antibodies E1/6 and Hu8/4 (IgG1), respectively, were used as purified IgG at 20 $\mu\text{g/ml}$ (18). Blocking anti-E-selectin antibodies H18/7 (19) and 7A9, the latter obtained from Walter Newman (Lukosite, Boston, MA), were used as purified IgG or F(ab')₂ fragments at 25 $\mu\text{g/ml}$. Blocking anti- α_4 -integrin (CD49d) antibody HP2.1 (Amac, Inc., Westbrook, ME) was used as purified IgG at 25 $\mu\text{g/ml}$. Function-blocking anti- β_2 -integrin TS1/18, clone #HB203 (American Type Culture Collection, Rockville, MD) was used as purified IgG at 20 $\mu\text{g/ml}$. Murine mAb K16/16 (IgG1) was used as an isotype-matched, nonbinding control. For adhesion assays under flow, HECs were treated with mAb for 30 min at 37°C and T cells were incubated for 15 min at 4°C for 15 min before use in assays. All mAbs were used at saturating concentrations as determined by indirect immunofluorescence flow cytometry.

Immunofluorescent Staining and Analysis of Cells. Untreated and TNF-treated HECs, and CHO-P-selectin cells were harvested nonenzymatically from the culture plates using HBSS containing 3 mM EDTA (15 min at 37°C, 5% CO₂) and stained with HPDG2/3 anti-P-selectin mAb followed by FITC-conjugated goat F(ab')₂ anti-mouse Ig (1:30 dilution; Caltag, San Francisco, CA) (5). Purified T cells were stained with phycoerythrin (PE)-labeled anti-CD4 and fluorescein isothiocyanate (FITC)-labeled anti-CD8, or PE-labeled anti-CD4 and FITC-labeled anti-CD3 (Caltag). T cells were also stained with anti-L-selectin (LAM1-3) as detailed (5). Single- and two-color cell fluorescence of 10,000 cells was determined by flow cytometry on a FACScan® (Becton Dickinson & Co., Mountain View, CA). 100% of the cells in each staining tube were gated.

Parallel Plate Flow Chamber Analysis of Endothelial-Lymphocyte Interactions. The flow chamber apparatus used in these studies has been described in detail (5, 20). Briefly, the chamber consists of two stainless steel plates separated by a silastic gasket. The flow field dimensions are 5 mm wide \times 30 mm long \times 0.250 mm high. Confluent endothelial cells, or CHO-P-selectin, L cell E-selectin or L cell VCAM-1 monolayers grown to \sim 90% of confluence on circular 25-mm glass coverslips (Fisher Scientific, Inc., Medford, MA) were placed in the flow chamber. Defined flow levels were applied by drawing media containing T cells (10⁶/ml) through the chamber using a syringe pump (model 44; Harvard Apparatus Inc., Natick, MA). Temperature was maintained at 37° by heating plates. The flow apparatus was mounted on an inverted microscope (Nikon Diaphot) and the entire perfusion period (10 min for each condition) was recorded on videotape by a video camera and VCR. Leukocyte adhesion was determined as previously detailed (5) and rolling velocities were determined using an automated PC-based image analysis software program (OPTIMAS; Bioscan, Inc., Edmonds, WA). Each antibody used was also included at saturating levels in the perfusion buffer.

Results and Discussion

TNF- α treatment of HECs induces expression of E-selectin and upregulated expression of VCAM-1 and ICAM-1 (4, 18, 19). TNF treatment also induced surface expression of P-selectin that was increased at 6 h and declined to baseline after 24 h as detected by indirect immunofluorescence and

flow cytometry with anti-P-selectin mAb (HPDG 2/3) (Fig. 1 A). Control secondary FITC-labeled IgG is shown for comparison and was identical to an isotype-matched K16/16 (IgG1) nonbinding control (for clarity, data not shown). In addition, CHO-P-selectin cells uniformly expressed levels of P-selectin (Fig. 1 B) that were similar to activated HECs (compare Fig. 1, A to B). CHO-P-selectin cells did not react with mAb directed to E-selectin (H18/7 or 7A9) or control isotype-matched nonbinding K16/16 (not shown). Similarly, murine L cells stably transfected with human E-selectin (L cell E-selectin) or VCAM-1 (L cell VCAM-1) uniformly expressed E-selectin or VCAM-1, respectively, at levels that were similar to those of 6-h, TNF- α -activated HECs (not shown), but did not react with anti-P-selectin, -E-selectin mAbs or isotype-matched nonbinding mAb K16/16.

Purified CD4⁺ T cells were 98 \pm 2% positive for both CD3 and CD4 (n = 14), and \geq 75% of cells expressed high levels of L-selectin (Fig. 2, A-C). These purified T cells were 69 \pm 16% CD45RO⁺ and 23 \pm 14% CD45RA⁺ and <1% of purified cells were class II MHC positive as determined by immunofluorescence flow cytometry. In addition, purified cells could be stimulated to incorporate [³H]thymidine and secrete IL-2 (as determined by the HT-2 bioassay) by incubation with PHA (10 $\mu\text{g/ml}$) or plate-bound anti-CD3 (OKT3) in the presence of irradiated blood mononuclear cell from the same donor. Unstimulated cells showed no significant proliferative or cytokine secretory activity.

CD4⁺ T cell adhesive interactions with 6-h TNF- α -activated HECs were examined under defined laminar flow using a previously described in vitro flow model (5). Substantial CD4⁺ T cell rolling and stable adhesion (arrest) occurred at 1.8 dynes/cm²; at a higher level of flow (4.4 dynes/cm²), few, if any, rolling or arrested cells were observed. In contrast, unactivated HEC monolayers did not support rolling interactions or arrest of CD4⁺ T cells at or above 1.8 dynes/cm².

Visual examination of CD4⁺ T cell interactions with the activated HECs revealed numerous T cells in contact with the apical surface of the endothelium. The initial contact consisted of freely flowing T cells abruptly coming to a complete halt on an endothelial cell surface. This contact was similar to that previously detailed for monocyte interactions with IL-4-activated HECs (5). Typically, each high-powered (\times 40) field contained \sim 20 T cells of which one third (36 \pm 6%) were rolling while the remaining cells were stably adhered. In these studies, arrested CD4⁺ T cells either: (a) subsequently began slowly rolling downstream on the apical surfaces of endothelial cells at 3.8 \pm 0.5 $\mu\text{m/s}$ (Table 1) for variable periods of time before stably arresting or releasing to the flow stream; (b) remained adherent and retained their round, spherical shape; (c) remained stably adherent for several minutes and then spread on the apical surface; (d) spread on the apical endothelial surface and migrated to intercellular borders before transmigrating beneath the monolayers; or (e) released to the flow stream. The video images in Fig. 3 depict the sequence of cellular events that leads to T cell transmigration across 6-h TNF-activated endothelial mono-

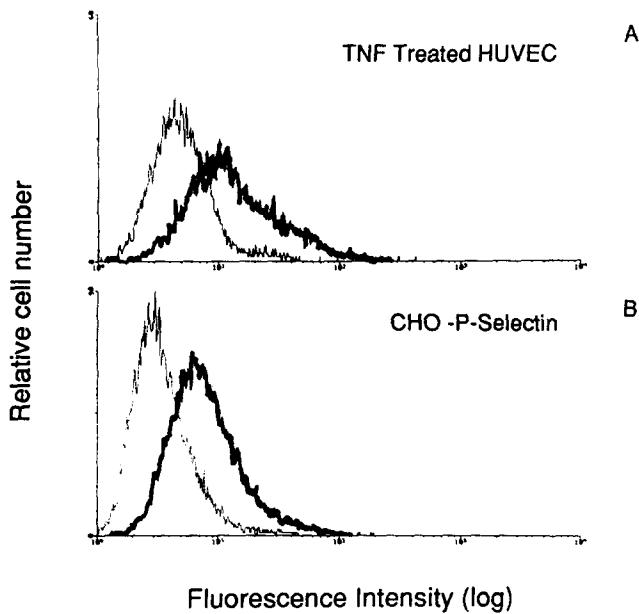


Figure 1. Surface expression of P-selectin on TNF- α -activated HEC and CHO cell monolayers. TNF- α -activated HEC monolayers (A) and CHO P-selectin cells (B) were analyzed for P-selectin expression by immunofluorescence and flow cytometry. Anti-P-selectin staining is shown by the heavy tracings and background staining by secondary FITC-labeled antibody is shown by the light tracings.

layers under laminar flow at 1.8 dynes/cm². In Fig. 3 *a*, a stably arrested T cell (*a*, arrow) begins to spread (*b*) before transmigrating beneath the endothelial monolayer (*c-e*). CD4⁺ T cell diapedesis usually occurred rapidly (minutes) once T cells had been stably arrested on the apical surface for several minutes. In addition, two rolling lymphocytes (arrowheads) enter the field from the right (Fig. 3, *b* and *c*, respectively) and roll across the monolayer (flow is from right to

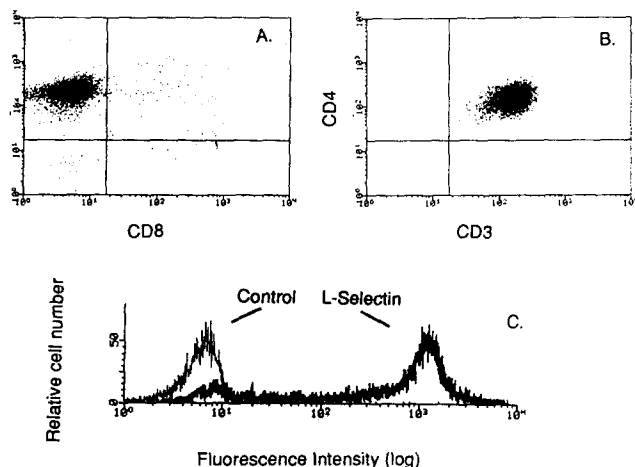


Figure 2. Phenotype of purified CD4⁺ T cells. T cells were stained with PE-labeled anti-CD4 and FITC-labeled anti-CD8 (A), or PE-labeled anti-CD4 and FITC-labeled anti-CD3 (B). T cells were also stained with anti-L-selectin (heavy tracing) or isotype matched control mAb (light tracing), followed by FITC-anti mouse Ig (C).

Table 1. CD4⁺ T Cell Rolling Velocities on TNF-activated HEC

mAb treatments	CD4 ⁺ T cell rolling velocities $\mu\text{m/s}$
Media	3.8 \pm 0.5
L-selectin (LAM1-14, control mAb)	4.3 \pm 0.6
L-selectin (LAM1-3 mAb)	4.8 \pm 1.1
P-selectin (HPDG2/1, control mAb)	3.9 \pm 1.4
P-selectin (HDPG 2/3 mAb)	6.1 \pm 1.3
E-selectin (H18/7 mAb)	3.6 \pm 1.7
VCAM-1 (Hu8/4, control mAb)	3.9 \pm 0.6
VCAM-1 (E1/6 mAb)	8.0 \pm 0.8*
$\alpha_4\beta_1$ -integrins (HP2.1 mAb)	5.9 \pm 0.6*
β_2 -integrins (TS1/18 mAb)	3.8 \pm 0.7
$\alpha_4\beta_1$ - and β_2 -integrins (HP2.1 and TS1/18)	6.8 \pm 1.1*

The velocities of CD4⁺ T cells rolling on TNF- α -activated HEC monolayers was determined at four separate time points after 9–10 min of perfusion by measurement of the distance CD4⁺ T cells traveled in 60 consecutive frames of videotape using an automated PC-based image analysis software program (OPTIMAS, Bioscan, Inc., Edmonds, WA). Data are mean \pm SEM; 20–40 individual cells for each mAb treatment were analyzed in two to three separate experiments.

* $p < 0.009$ versus media, or control mAb LAM1-14 and Hu8/4.

left). The lymphocyte entering in *c*, which is lower in the field, rolls at a higher velocity than the lymphocyte that enters in *b*. Observation under a $\times 40$ objective clearly indicated that CD4⁺ T cells were rolling and not sliding or tumbling; the large nucleus rotated as the cell traveled across the HEC surface. The patterns of several selected CD4⁺ T cells rolling on activated HECs are shown in Fig. 4 and demonstrate the variable behavior of rolling T cells. Certain T cells rolled at steady velocities, either fast or slow, while others rolled for a few seconds before stably arresting again or releasing to the flow stream. This behavior was consistently observed in this system in several separate experiments. Others have reported similar behavior for lymphocytes interacting with endothelium in high endothelial venules (HEV) in situ (21, 22).

We next investigated the mechanisms that mediated these adhesive interactions using function blocking mAb under flow at 1.8 dynes/cm². Inhibition of L-selectin by function blocking mAb (LAM1-3) did not reduce the number of adherent CD4⁺ T cells adhesion (Fig. 5) or their rolling velocities (Table 1). Other blocking mAb to L-selectin (LAM1-4, DREG-56) also had no inhibitory effect (not shown). Control nonblocking isotype-matched mAb directed to L-selectin (LAM1-14) had no effect. In addition, PMA stimulation (100 ng/ml, 15 min at 37°C) of CD4⁺ T cells did not alter their adhesion to TNF-activated HEC monolayers (unactivated T cells, 357 \pm 168 cells/mm² versus PMA-activated, 359 \pm 125 cells) even though this treatment caused near total loss

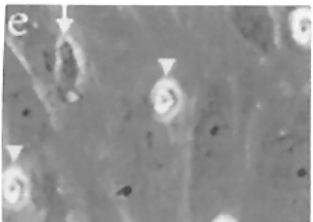
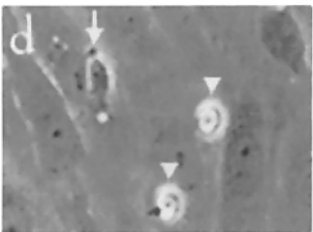
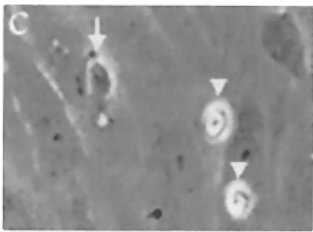
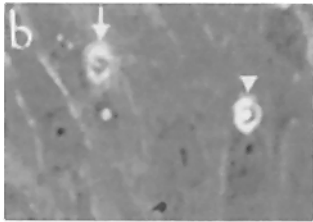
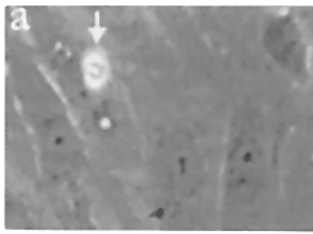


Figure 3. CD4⁺ T cells roll on TNF- α -activated HEC monolayers under flow. Confluent TNF- α activated (6 h, 50 ng/ml) monolayers were inserted into the flow chamber and CD4⁺ T cells were perfused across the monolayer at 1.8 dynes/cm² (0.9 ml/min) as previously detailed (5). Flow is from right to left. Digitized video images of CD4⁺ T cells rolling on HEC monolayers were obtained 14, 23, 5, and 10 s apart ($\times 40$). The arrow indicates a cell that arrests (a), spreads (b), and then transmigrates (c-e). Two lymphocytes rolling at different velocities are indicated by arrowheads (b-e).

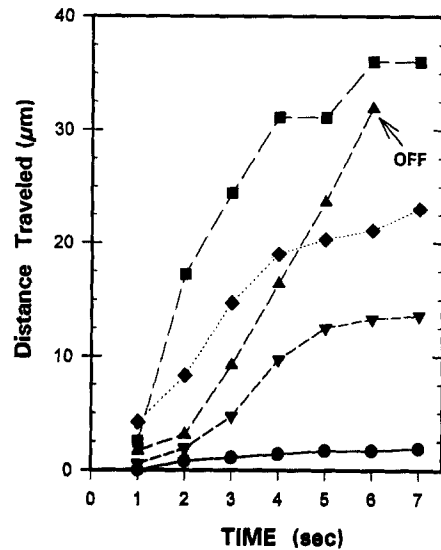


Figure 4. Behavior of rolling CD4⁺ cells during interactions with 6-h TNF- α -activated HEC monolayers under flow at 1.8 dynes/cm². The traces of selected CD4⁺ T cells illustrate their variable rolling behavior on HEC under flow. In particular, one cell (solid triangle) rolls at ~ 8 μ m/s for 6 s before releasing to the flow stream (OFF).

of L-selectin expression. Treatment with anti-E-selectin mAb H18/7 reduced adhesion in two of nine separate experiments, however, overall H18/7 did not significantly reduce adhesion. Another function-blocking mAb to E-selectin (mAb 7A9) also did not reduce adhesion (data not shown) in two experi-

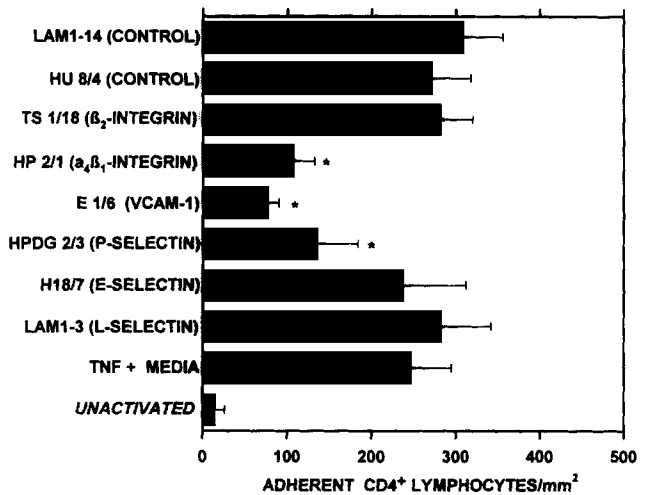


Figure 5. Monoclonal inhibition of CD4⁺ T cell adhesion on TNF- α -activated HEC monolayers. Confluent TNF- α -activated (6 h, 50 ng/ml) or control HEC monolayers were washed three times, incubated with indicated mAb at saturating concentrations (see Materials and Methods) for 30 min at 37°C, and inserted into the flow chamber. Concomitantly, CD4⁺ cells were incubated with appropriate mAb for 15 min on ice, diluted to 12 ml and perfused across monolayers at 1.8 dynes/cm². Firmly adherent cells were determined (12–15 fields at $\times 40$) after 9–10 min of perfusion (rolling cells were not included). $N =$ five to nine separate experiments for each mAb treatment; unactivated, $N = 15$ and TNF- α + media, $N = 15$. * $p \leq 0.025$ by paired Student's t test.

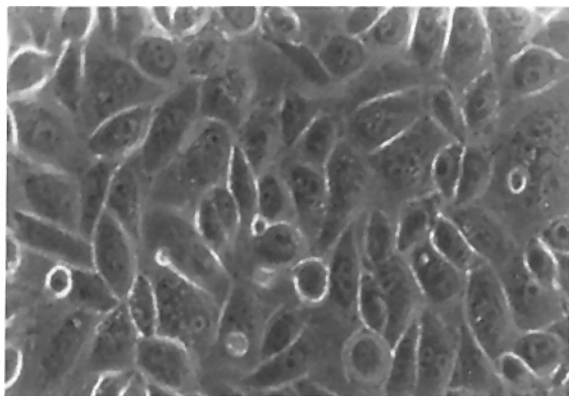
ments. Thus, in one experiment where H18/7 reduced adhesion on activated HECs, several CD4⁺ cells per field engaged in rolling interactions with L cell E-selectin monolayers, whereas in three other experiments in which H18/7 had no effect, few, if any CD4⁺ T cells interacted with L cell E-selectin monolayers. Although previous studies have shown that T cells bind to E-selectin (1, 23, 24), this population of lymphocytes appears to constitute a subset of circulating memory T cells (referred to as "skin homing" [24]) which varies with each blood donor, and, therefore, may have been too small to detect in all T cell preparations in our assay. In contrast, anti-P-selectin mAb (HPDG 2/3) reduced initial attachment of T cells and thus reduced adhesion by 50% ($p \leq 0.007$; $n = 8$). A control isotype-matched nonblocking anti-P-selectin mAb HPDG 2/1 had no significant effect on adhesion (389 ± 103 , $n = 2$) or rolling velocities (Table 1). Although inhibition of P-selectin by mAb HPDG 2/3 increased rolling velocities (Table 1), the difference was not significant ($p > 0.13$). This is not surprising since the initial attachment and thus adhesion of up to 50% of T cells was independent of P-selectin and the rolling velocities of these cells were averaged into the determination. This result suggests that the P-selectin mAb inhibition was incomplete or that other adhesion molecule(s) are operative in this system. The combination of anti-P-selectin with blocking L-selectin mAb was not more effective than anti-P-selectin mAb alone ($\sim 50\%$ inhibition; $p \leq 0.025$, $n = 2$). Furthermore, the combination of mAb directed to E-, L-, and P-selectin was not more effective than P-selectin alone. As further evidence for P-selectin involvement, we examined CD4⁺ T cell interactions with CHO-P-selectin monolayers (Fig. 6). The photomicrographs in Fig. 6 depict the movements of five different CD4⁺ T cells on CHO-P-selectin monolayers treated with control mAb H18/7 (nonbinding). Three CD4⁺ T cells were rolling along the apical surface at different velocities (arrows, *Subtracted Image*), while two other T cells remained stationary (arrowheads). The average rolling velocity of 40 cells rolling on CHO-P-selectin was $38 \pm 18 \mu\text{m/s}$ (mean \pm SD, $n = 3$ experiments). In general, T cells interactions consisted of cells rolling and transiently adhering for a few seconds before rolling again. No stably adherent cells (adherent for >10 s) were observed. mAb HPDG 2/3 mAb totally abolished CD4⁺ T cell interactions, whereas control K16/16, anti-E-selectin (mAb H18/7) and nonblocking HPDG 2/1 had no effect. We note here that both CHO-P-selectin and L cell E-selectin monolayers supported both human monocyte and neutrophil rolling at 1.8 dynes/cm²; appropriate function blocking mAb totally abolished leukocyte rolling (our unpublished observations).

$\alpha_4\beta_1$ integrins interacting with endothelial-expressed VCAM-1 was the predominant mechanism that mediated arrest of rolling CD4⁺ T cells, whereas β_2 -integrins were involved in spreading and transmigration under flow. mAb directed to either $\alpha_4\beta_1$ (HP2.1 mAb) or VCAM-1 (E1/6) blocked 61 and 75% of adhesion (Fig. 5), respectively, and both mAbs significantly increased the rolling velocities (Table 1). Inhibition of this pathway resulted in an increase in both

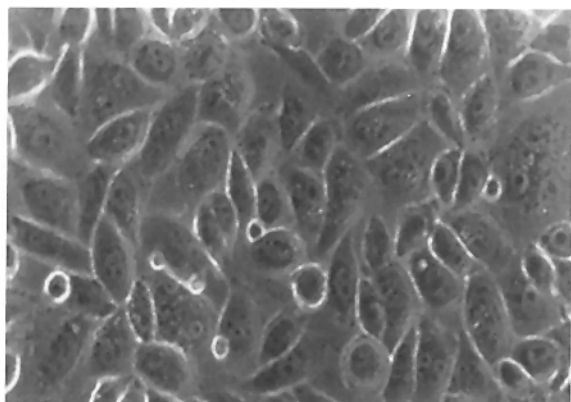
the number of rolling cells, since T cells were unable to stably arrest, and their rolling velocities (Table 1). These data suggest that rolling is mediated through overlapping functions of P-selectin and VCAM- $\alpha_4\beta_1$ pathways under flow. The ability of VCAM- $\alpha_4\beta_1$ pathway alone to mediate arrest or support rolling under flow was further examined using L cell VCAM-1 monolayers. At 1.8 dynes/cm², CD4⁺ T cell rolling on VCAM-1 L cell monolayers was an extremely rare event characterized by a transient tumbling motion of the T cells followed by release. Stable arrest (>10 s) was not observed. Thus, $\alpha_4\beta_1$ -VCAM-1 interactions were not sufficient to mediate rolling or arrest of unactivated CD4⁺ T cells at 1.8 dynes/cm² in this system. In contrast, anti- β_2 -integrin mAb (TS1/18) did not significantly alter adhesion or rolling velocities. However, mAb to β_2 -integrins reduced lymphocyte spreading and/or transmigration across activated HECs by 53% ($15 \pm 6\%$ cells spread/transmigrated, $p \leq 0.0002$, $n = 2$) as compared with anti- $\alpha_4\beta_1$ or nonblocking anti-L-selectin (LAM1-14) mAb (39 ± 11 and $32 \pm 10\%$, respectively). Furthermore, when both β_1 - and β_2 -integrins were simultaneously blocked with mAbs, nearly all T cells were rolling and few T cells arrested (25 ± 25 cells/mm²), and the rolling velocities were increased significantly as compared with control treatments (Table 1). This is consistent with in vivo studies suggesting involvement of both β_1 - and β_2 -integrins in peripheral T cell recruitment to sites of immune reactions (25).

Several features of our analyses of CD4⁺ T cell interactions with endothelial monolayers under flow merit emphasis. First, almost 40% of CD4⁺ T cells in contact with the activated endothelial monolayer rolled on TNF- α -activated endothelium under flow. The rolling velocities of CD4⁺ T cells were about twofold less than that reported for neutrophils rolling on TNF- α -activated HEC under flow at 1.85 dynes/cm² (10). Interestingly, the pattern of adhesion of the lymphocytes to TNF-activated endothelium was different from that previously reported for monocytes (5). Specifically, once a lymphocyte stably arrested, there was no preferential attachment of other lymphocytes just downstream from the first cell, but rather, attachment appeared to be random. Secondly, P-selectin mediated a significant proportion of CD4⁺ T cell initial contact, and thus facilitated $\alpha_4\beta_1$ -integrin-dependent arrest, whereas E- and L-selectin were not involved in this system. Third, CHO-P-selectin monolayers supported CD4⁺ T cell rolling and transient adhesion under flow at 1.8 dynes/cm². That the T cell rolling velocities were significantly greater on CHO-P as compared with activated HECs suggests that other molecule(s) in addition to P-selectin participate in T cell rolling on activated vascular endothelium. P-selectin has recently been implicated in mediating spontaneous leukocyte rolling in vivo (26), in monocyte recruitment to inflamed synovium (27), in leukocyte rolling and neutrophil extravasation at acute sites of inflammation (28), and finally, immobilized P-selectin chimera molecules support CD4⁺ T cell adhesion under static conditions (29). Fourth, surprisingly, L-selectin was not significantly involved, even though we (16) have reported previously that mAb

FRAME 1



FRAME 2



Subtracted Image

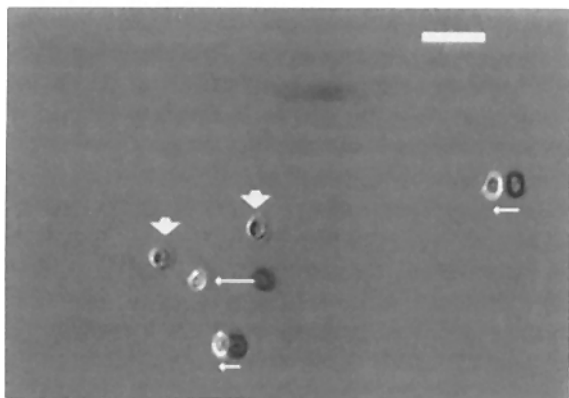


Figure 6. Adhesion and rolling of CD4⁺ T cells on CHO P-selectin monolayers under flow at 1.8 dynes/cm². The movements of five different T cells on CHO P-selectin monolayers treated with H18/7 mAb (control mAb) are shown. Two frames of videotape from a single experiment were digitized (*FRAMES 1* and *2*). Frame 1 (*top*) was then subtracted from Frame 2 (*middle*) to yield the subtracted image (*bottom*). In the subtracted image, the dark spots represent the original position of three lymphocytes and the bright spots indicate their new location 1 s later. The horizontal arrows depict the path of cell movement while the vertical arrowheads

blockade of L-selectin inhibited ~40% of unfractionated peripheral blood lymphocyte adhesion to TNF- α -activated HECs under rotating conditions. This difference may reflect the use of highly purified CD4⁺ T-cells in this study as well as the use of simulated blood flow conditions. Fifth, our data demonstrate that $\alpha_4\beta_1$ -VCAM-1 interactions are the predominant mechanism that mediates T cell arrest under flow. Our attempts to directly show that the $\alpha_4\beta_1$ -VCAM-1 pathway mediates T cell rolling at 1.8 dynes/cm² using VCAM-1-transfected L cells were largely unsuccessful. However, the observation that mAb blockade of this pathway significantly increased lymphocyte rolling velocities implies its involvement in the process of rolling. This may reflect both the adhesive functions of this ligand pair and also possible T cell signalling events mediated by $\alpha_4\beta_1$ (30, 31). Taken together with the inhibitory affect of P-selectin mAb, the data suggest that P-selectin is involved in T cell initial contact and subsequent rolling on TNF-activated HECs, and that both P-selectin and VCAM-1- $\alpha_4\beta_1$ pathways function in an overlapping manner to support CD4⁺ rolling at very low velocities before stable arrest. Finally, our findings that a well-characterized function blocking mAb of β_2 -integrins inhibited only 50% of T cell spreading/diapedesis and did not affect rolling or arrest suggest that a significant component of T cell recruitment is not dependent on β_2 -integrins. The fact that cell-mediated immune reactions are normal in leukocyte adhesion deficiency type I (LAD I) patients is consistent with this finding.

T cell interactions with lymphoid HEV are relatively well-characterized (4, 21, 22). In contrast, the mechanisms of T cell recruitment to peripheral sites of inflammation are not well understood. These studies include a detailed molecular analysis of CD4⁺ T cell recognition of endothelium and indicate a requirement for sequential and overlapping actions of P-selectin with β_1 - and β_2 -integrins, respectively, during rolling, arrest, and diapedesis. Many immune-mediated inflammatory conditions depend on helper T cell adhesion and transmigration through cytokine-activated vascular endothelium at peripheral sites of antigen exposure. Therefore, our data suggest that blockade of β_1 -integrin-VCAM-1 interactions and P-selectin interacting with its leukocyte ligands may have therapeutic benefits in autoimmune diseases and allograft rejection.

identify two cells that remained stationary. Pretreatment of CHO P-selectin monolayers with blocking HPDG 2/3 totally abolished these interactions (not shown). Photographs were digitized from frames of videotape, the contrast and brightness adjusted by software (Photoshop v2.5, Adobe) and printed on a digital printer (Tektronix IISDx). Bar, 20 μ m.

We thank Drs. Thomas Tedder for LAM1 mAb, L cell VCAM-1 and L cell E-selectin cells, Dale Cumming, for P-selectin mAb and CHO-P-selectin cells, Eugene Butcher for mAb DREG 56, Walter Newman for 7A9 mAb, and Geoffrey Kansas (Northwestern University, Evanston, IL), Michael A. Gimbrone, Jr., and other members of the Vascular Research Division for fruitful discussions.

This work was supported by National Institutes of Health grant HL 36028.

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Received for publication 27 October 1994.

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